# NEW METHOD FOR BIODEGRADABILITY OF COLLAGEN AND KERATIN BASED MATERIAL ASSESSMENT

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Biodegradability of widely-used materials has become an important environmental property and a market tool for high quality articles intended for responsible consumers. Assessing biodegradability of natural furskins is a difficult endeavour due to the complex composition of natural fur skins. Originality of research consists in elaborating a method of assessing biodegradability of sheep furskins based on specific microorganisms for collagen and keratin degradation. In this regard, preparations with collagenase and keratolytic activity were developed and characterized by determining enzymatic activity before and after concentration and conditioning. Strains were identified using BIOLOG – Microbial Identification system. Experiments on biodegradation of natural furskins were performed in the WTW – OxiTop device and have enabled assessment of biodegradability by determining biochemical oxygen demand every 3 days for a 45-day period. Selected strains have shown ability to hydrolyze furskins at the end of their life cycle. The elaborated method enables biodegradability assessment of natural furskins in a shorter span of time compared to similar methods for plastics, which take minimum 6 months, and require much higher costs.

Keywords: biodegradability, furskin, collagen, keratin, enzymatic activity, specific enzymes.

## INTRODUCTION

The research on biodegradability assessment of natural leather is of high interest as a scientific tool to eliminate the misunderstanding regarding the commercially "ecological" synthetic furskins and leathers. The recent discussions regarding the need for supporting through EU legislation the labeling of natural leather authenticity of leather goods, garment and upholstery (international Leather Maker, 2014) represent another tool for consumer education and protection. The development of a method for biodegradability assessment of natural leathers and furskins represents another instrument for leather goods labeling and consumer perception improving in connection to sustainable environmental protection as compared to synthetic materials with less durability and non biodegradability.

The definition (van der Zee, 2011) of polymers biodegradability and assessment was delimited from the other meanings as a process which best simulates the disposal pathway, keeps the accumulation under control, generates as end products $CO_2$, water, minerals, intermediate biomass which includes humic materials and the polymers biodegrade safety or the use of end products must be friendly.

Even though there are enough standards for polymer biodegradability testing many works need to be done to understand the mechanism of biodegradation. The lack of methods for the survey of intermediate materials generated by biodegradation is the most important blocked progress in biodegradability assessment.

The recent literature reports the biodegradability assessment of wet-white, vegetable or chromium tanned leathers (Qiang *et al.*, 2011). Composting for 150 days of leathers tanned with different organic phosphates, vegetable tannin and chromium salts showed a rate of degradation between 6-80% depending on crosslinking strength. The influence of leather manufacturing technology and consequently of the leather composition on biodegradability is stressed in different studies. The first tannery who claims to be the most ecological and certified for the first time a biodegradable product is Dutch Hulshof with Piuro product, a fully organic product (Hulshof).

The efforts to adapt the methods for plastics biodegradability assessment to leathers were already reported and consist in measurement of $CO_2$ released by biomass biodegradation (Bertazzo *et al.*, 2011; Calise, 2011; Lombardi, 2013). Even under these conditions the test is time-consuming and needs a step forward in microorganism selection and rapid assessment of biodegradability of leathers. The first step in biodegradability assessment is the evaluation of structural changes of materials under the composting conditions and understanding the mechanism of biodegradation initiation.

Furskins are complex materials composed of keratin and collagen, two proteins with different reactivity, and literature does not report any study regarding the biodegradability of furskins. Even the processing of furskins must comply with fashion trends and chemical materials are very complex (from syntans to finishing binders), the main components are collagen and keratin, biodegradable materials. In comparison to synthetic leathers and furskins, wrongly called “ecological”, natural furskins are more durable and less pollutant materials.

The paper presents some progresses in bacterial preparation for sheepskin biodegradability evaluation and a proposed method for biodegradability assessment.

## MATERIAL AND METHODS

### Selection of Bacterial Preparations Specific to Fur Substrates

To screen microorganisms specific to fur substrates two different sources were taken into account; the first was to isolate them from natural environments, and the second was the microorganism collection of the Microbial Biotechnology Centre (BIOTEHGEN).

In the experiment four bacteria strains were used, marked: BN7, Bl, 7.2 and Omf. Strains BN7, 7.2 and Omf are isolated naturally from the soil, and Bl is a strain from the collection (Bacillus licheniformis ATCC 14580).

To identify how these bacterial strains act on natural furs, they were grown in a minimal medium with pH=7.5 with the following composition: NaCl 1 $gL^{-1}$; $CaCl_2$ 0.05 $gL^{-1}$; $KH_2PO_4$ 0.7 $gL^{-1}$; sucrose 3 $gL^{-1}$; $MgSO_4$ 0.9 $gL^{-1}$; $K_2HPO_4$ 2.38 $gL^{-1}$, with fur samples as source of carbon and nitrogen. Bacteria strains were grown at 30-32°C under stirring (140 rpm).

### Determining Collagenase and Keratinase Activity of Bacterial Preparations

Samples were analyzed in terms of enzymatic activity (EA) of collagenase and keratinase, as well as soluble protein content.

The method of determining collagenase activity is based on hydrolysis of collagen under the action of collagenase, as a result of which peptides are released. The degree of proteolysis is measured spectrophotometrically at 570 nm in the presence of ninhydrin.

Determination results were expressed in U/ml. One unit of collagenase is the amount of enzyme which releases peptides from collagen equivalent in color intensity determined spectrophotometrically using ninhydrin with 1.0 µmole of leucine in 5 hours at pH 7.4 and temperature of 37°C in the presence of calcium ions.

The method of determining keratinase activity is based on hydrolysis of the substrate (keratin azure) under the action of keratinase. The change in absorbance at 595 nm due to the intensifying coloration by substrate hydrolysis is determined spectrophotometrically. Determination results were expressed in U/ml. A keratinase unit is the amount of enzyme which causes an increase in absorbance of 0.01 at 595 nm after one hour reaction at 60°C while stirring at 150 rpm.

**Identifying Bacteria Strains Using BIOLOG – Microbial Identification System**

To identify bacterial strains isolated from natural environments, marked B7.2, BN7 and Omf using BIOLOG - Microbial Identification system the steps were the following: strains were inoculated on BUG agar medium and incubated for 24 hours at 33°C; the device was calibrated by densimeter calibration and verification of inoculum medium density; medium was inoculated with a bacterial colony of 84-89% density; GENIII plates were inoculated with 100ul of the inoculum; and incubated at 33°C for 20-22 hours and results were read using the Biolog spectrophotometer.

**Concentration of Bacteria Preparations**

In order to increase the stability of microbial enzyme preparation, the process of lyophilization was applied (freeze-drying under vacuum) to strains B7.2, BN7 and Bl. Lyophilization consisted in adding 10% trehalose as cryoprotectant agent, freezing the samples and then lyophilization (Labconco freeze dryer) at a pressure of 0.04 mbar, at temperatures between -40 and 45°C for 18 hours. Lyophilized powder reconstitution was performed using sterile distilled water.

**Biodegradability Assessment of Natural Furs**

Incubation is done in the dark or dim light in brown pots sterilized at constant temperature of 35°C ± 2°C under magnetic stirring in the WTW - OxiTop device on finished sheep furskin samples. At least two samples of fur are inserted into vessels in sterilized water with the bacterial preparation and two control samples without bacterial preparation. Any toxic contaminants or inhibitors for enzyme preparations are avoided and all working vessels and manipulation tools are carefully sterilized.

Biochemical oxygen demand (BOD) is automatically measured in the range 0-400 mg/L starting with time 0 and every 3 days for at least 45 days. The degree of biodegradability is calculated as follows:

$$B\% = (x\ mgO_2/L : 360\ mgO_2/L)x100 \quad (1)$$

where: x is the amount of $O_2$/L released after biodegradation of fur; 360 is the theoretical amount of $O_2$ required for complete decomposition of 0.6 g fur with 11% moisture in $CO_2$ (ThOD).

The test is considered valid if the control vials consume less than 20% of the oxygen demand consumed by the test sample.

## RESULTS AND DISCUSSIONS

### Collagenase and Keratinase Activity of Bacterial Preparations Specific to Natural Furskins

The test results on collagenase and keratinase activity of selected bacterial preparations are summarized in Table 1.

Table 1. Activity of the enzymatic complex synthesized by bacterial strains on natural furskins

| Sample | Protein (mg/ml) | Collagenase EA (U/ml enzyme) | Keratinase EA (U/ml enzyme) |
|---|---|---|---|
| B7.2 | 1.59 | 14.56 | 2.76 |
| BN7 | 1.45 | 18.07 | 2.54 |
| Omf | 1.58 | 13.13 | 1.08 |
| Bl | 1.42 | 23.7 | 4.44 |

In samples containing sheep furskin amounts of protein were determined between 1.42 - 1.59 mg/ml, indicating that the microorganisms synthesize their required amount of protein from carbon and nitrogen sources provided. The highest collagenase activity was recorded for Bl strain in samples containing sheep furskin (23.7 U/ml enzyme). Another strain with remarkable collagenase activity, of 18.07 U/ml enzyme, was BN7.

The concentration of the bacterial enzyme complexes has led to the results shown in Table 2.

Table 2. Activity of the enzymatic complex in concentrated enzymatic preparations

| Initial sample | Protein (mg/ml) | Collagenase EA (U/ml enzyme) | Collagenase SEA (U/mg protein) | Keratinase EA (U/ml enzyme) | Keratinase SEA (U/mg protein) |
|---|---|---|---|---|---|
| BN 7 | 1.28 | 14.44 | 11.27 | 2.45 | 1.92 |
| BI | 0.89 | 16.33 | 18.21 | 5.09 | 5.67 |
| B7.2 | 0.57 | 3.7 | 6.49 | 0.39 | 1.46 |
| Lyophilized sample | Protein (mg/ml) | Collagenase EA (U/ml enzyme) | Collagenase SEA (U/mg protein) | Keratinase EA (U/ml enzyme) | Keratinase SEA (U/mg protein) |
| BN7 | 0.97 | 20.49 | 21.06 | 2.96 | 3.04 |
| BI | 0.76 | 17.81 | 23.53 | 4.69 | 6.19 |
| B7.2 | 0.52 | 3.28 | 6.3 | 0.37 | 1.72 |

In Table 2, Bl strain stands out, with the highest initial specific collagenase activity (18.21 U/mg protein), which increased 1.3 times by lyophilization as a result of concentration processes it has been subjected to.

Good specific collagenase activity was also determined for initial BN7 strain (11.27 U/mg protein), which increased 1.87 times by lyophilization.

### Identifying Bacterial Strains Isolated from Natural Environments

BIOLOG – Microbial Identification system is an advanced system for the identification and characterization of microorganisms, which allows rapid identification

of over 1,900 species of aerobic and anaerobic bacteria, pathogenic bacteria, filamentous fungi (500 species) and yeasts (250 species), as well as analysis of microbial communities. GENIII BIOLOG system is based on the analysis of metabolic pattern resulting from the breakdown of the major classes of biochemicals by microbial cells (71 carbon sources) and determination of important physiological properties such as pH, tolerance to salinity, acidity, and testing sensitivity to various chemicals.

Three bacterial strains isolated from natural environments, marked B7.2, BN7 and Omf were identified using BIOLOG – Microbial Identification system. To identify strains, the following are taken into account: index of similarity value (SIM), which is a comparison between the results of the biochemical characteristics of the tested bacterial strain and biochemical test results obtained with strain collection (ATCC) used to obtain BIOLOG databases and the distance (DIST), which is a comparison between the first two results obtained. For a conclusive identification it is necessary that SIM>0.5, and DIST value should be at least 2 (Table 3). Figure 1 shows how to identify strains isolated from natural sources on GENIII plates.

Table 3. Identification of bacterial strains using the BIOLOG system

| Samples | Strain | Similarity | Distance |
|---|---|---|---|
| B7.2 | Bacillus amyloliquefaciens | 0.603 | 5.814 |
| BN7 | Bacillus amyloliquefaciens | 0.589 | 6.006 |
| Omf | Bacillus amyloliquefaciens | 0.575 | 6.230 |

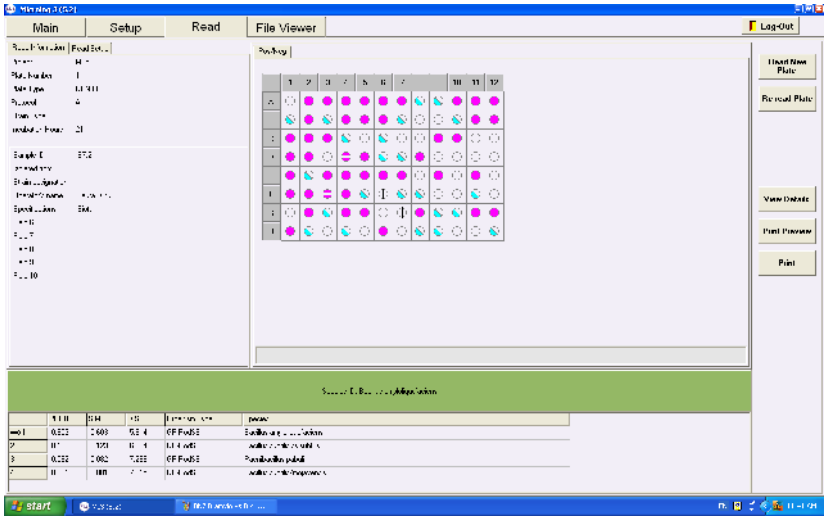


Figure 1. Identification of B7.2 strain on GENIII plate

**Biodegradation of Natural Furskins**

The results of the accelerated biodegradation of sheep furskin samples are presented in Figure 2. Figure 2 clearly shows a significantly higher oxygen consumption in the case of inoculum based on BI with the maximum value of 363 mg/L (100% biodegradable), while other types of inoculi generate consumption between 75-220 mg/L, the control shows a BOD value of 2.8-16.9 mg/l.

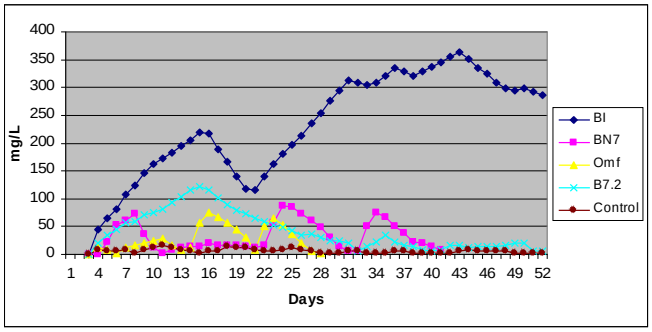


Figure 2. BOD of the sheepskins incubated with BI, BN7, Omf, B7.2 as compared to the control sample

## CONCLUSIONS

Enzymatic preparations based on Bacillus licheniformis ATCC 14580, grown in specific environments for furskins and concentrated by lyophilization allow accelerated biodegradation of natural fur and are a useful material for the development of methods to assess biodegradability of natural fur. The research paves the way for the development of enzymatic preparations allowing faster evaluation of biodegradability of leather or fur in order to increase confidence in the environmental value of natural products.

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